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PRINCIPAL INVESTIGATOR: Dr. Eva Top

CONTRACTING ORGANIZATION: University of Idaho
Moscow, ID 83844-3051

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14. ABSTRACT Plasmids play an important role in the spread of genes that confer resistance to antibiotics among bacterial pathogens. Given the worrisome rise of bacterial multi-drug resistance worldwide, the goal of our current research project is to characterize the evolutionary mechanisms by which multi-drug resistance (MDR) plasmids improve their persistence in biofilms formed by important wound pathogens. The central hypothesis of this study is that the evolutionary pathways through which stable plasmid maintenance improves are different and more varied in biofilms than in well-mixed liquid cultures due to the uniquely spatially structured environment of biofilms. To address this hypotheses the following aims are being addressed: (i) Compare the persistence of MDR plasmids in populations of clinically relevant bacteria grown in biofilms and well-mixed liquid cultures; (ii) Compare the evolution of plasmid persistence in bacteria grown in biofilms and well-mixed liquids; (iii) Characterize evolutionary changes that occur during stabilization of plasmid-host pairs under both conditions. During the first year of this two-year study, we accomplished several aspects of the first two tasks outlined in the SOW. Our preliminary results indicate that the structured biofilm environment, which typically characterizes the type of bacterial growth in wounds, facilitates the persistence of MDR plasmids in <i>Acinetobacter baumannii</i> , a problematic wound pathogen. Moreover, we have shown that plasmids coevolved with their host under antibiotic selection for 50 days have improved their persistence in part by undergoing large structural changes. While still partially uncharacterized, these genetic modifications likely decrease plasmid cost to the host while retaining favorable plasmid-encoded traits (i.e. antibiotic resistance). Through whole genome resequencing we will further characterize these structural changes and other possible mutations in these evolved populations as well as in populations evolved in biofilms. His project will greatly contribute to our understanding of the pathways through which plasmid persistence can improve in <i>Acinetobacter baumannii</i> and other wound pathogens when grown in biofilm environments. This project has the potential of supporting future research on therapeutic agents targeting maintenance and spread of MDR plasmids. Such therapies will ultimately be useful in the care of patients with combat-related wound infections.					
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1. INTRODUCTION:

Infections of wounds sustained during combat may compromise an individual's survival by leading to septicemia. This process is complicated if the infection is caused by multi-drug resistant (MDR) Gram-negative bacteria that form biofilms in the wounds, such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Enterobacter sp.*, and *Escherichia coli* (Eardley *et al.*, 2011; Gaynes & Edwards, 2005; Murray, 2008). Plasmids play an important role in the spread of genes that confer antibiotic resistance among bacterial pathogens. In spite of the worrisome rise of bacterial multi-drug resistance worldwide, it is still not known how resistance plasmids are successfully maintained in the absence of antibiotic selection in bacterial populations that grow as biofilms. Therefore, understanding the genetic mechanisms involved in the evolution of plasmid stability in biofilms is critical in our search for targets for alternative therapeutic approaches. The goal of this research project is to characterize the evolutionary mechanisms by which multi-drug resistance (MDR) plasmids can improve their persistence in biofilms formed by various Gram-negative bacteria. The central hypothesis of this study is that the evolutionary pathways through which stable plasmid maintenance improves are different and more varied in biofilms than in well-mixed liquid cultures due to the uniquely spatially structured environment of biofilms. This research project has the potential to support future research on therapeutic agents targeting maintenance and replication of MDR plasmids (Baquero *et al.*, 2011). Such therapies will ultimately be useful in the care of patients with combat-related wound infections, which would have otherwise have been difficult to treat.

2. BODY of REPORT:

The successful spread of a multi-drug resistance (MDR) plasmid is measured in terms of its ability to replicate, persist, and horizontally spread in a given bacterial population or community. We hypothesize that one of the major factors affecting plasmid persistence is the spatial structure of the environment. In well-mixed liquid batch cultures, typically used in the laboratory to study microbial evolution, newly arising mutants with beneficial mutations can rapidly increase their proportions in the population. This is due to their ability to easily outcompete their ancestral host and other less fit mutants in this well-mixed system. Such take-over of a mutant is called a 'sweep'. In contrast, the same mutants may not readily sweep through the bacterial population in a spatially structured environment such as a bacterial biofilm, because competition is local and not global. This provides opportunity for other, potentially fitter, mutants to arise and accumulate in the biofilm. As described in the SOW, we have proposed to conduct four Tasks that will allow us to better understand the mechanisms underlying evolution of plasmid persistence in biofilms as compared to liquid mixed cultures.

During the first year of this project, we have successfully accomplished parts of Tasks 1 and 2. We have made substantial progress in the characterization of the persistence of our two model broad-host-range MDR plasmids pRGM1 and pB10, particularly in *Acinetobacter baumannii*. A comprehensive description of accomplishments and challenges that we have encountered up to date is described here for each of our proposed Tasks.

Task1: Compare the persistence of naturally occurring multi-drug resistance (MDR) plasmids in populations of Gram-negative bacteria grown in biofilms and well-mixed liquid cultures.

This task is in turn divided into four *sub-tasks*:

1.a. Marking of plasmids with the *gfp* gene.

The characterization of the pathways by which MDR plasmids can become stable in structured biofilm and in liquid bacterial cultures is a complex process that involves multiple steps. We developed a high throughput method to measure plasmid presence and stability over time in multiple lineages simultaneously.

Making plasmid constructs

We used transposon mutagenesis to mark our two MDR broad-host-range model plasmids, the IncU plasmid pRGM1 and the IncP-1 plasmid pB10, with mini-Tn5-PA1-04/03::*gfp*, which contains a gene that encodes a green fluorescent protein (GFP). Plasmid pB10 was marked previously (Van Meervenne *et al.*, 2013), and in Year 1 we have also successfully inserted the same mini-transposon into pRGM1, although this process took longer than expected (see the ‘Problem areas’ section below for more detail).

Results

The process of transposon marking resulted in several pB10::*gfp* variants. We chose one that exhibited a bright fluorescent phenotype and determined that the mini-transposon was inserted in the gene *kfrC*. GFP expression can impose a fitness cost on the host (*i.e.*, it may decrease the host’s growth rate). Plasmid cost in turn affects its persistence in the population, as cells harboring a more costly plasmid will be more rapidly outcompeted by plasmid-free cells than by cells with a less costly plasmid (De Gelder *et al.*, 2007). To determine whether the insertion of the transposon affected plasmid stability we compared the stability of marked and unmarked plasmid in *Acinetobacter baumannii* ATCC 17978. So far we have focused on this strain as our main model host (from here on often abbreviated as *A. baumannii*). Plasmid stability in liquid cultures was determined as described by us previously (De Gelder *et al.*, 2007), except that fluorescence

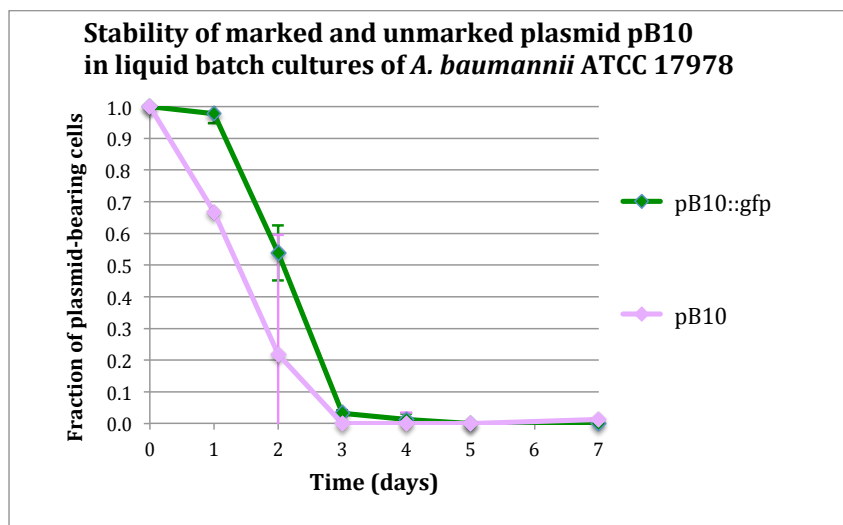


Figure 1: Comparison of the stability of pB10 and pB10::*gfp* in *A. baumannii* ATCC 17978 based on plate counting and replica-plating.

was now also used as a measure for plasmid presence in addition to antibiotic resistance. Thus for the marked plasmid, the numbers of fluorescent (putative plasmid-containing) and non-fluorescent (putative plasmid-free) colony forming units (CFU) were enumerated on LB agar plates containing 100-200 CFUs per plate, using a blue light source (450 nm). As shown in Fig. 1, the *gfp*-marked plasmid was at least as stable as the unmarked plasmid, suggesting that the marking would not have a significant effect on the outcome of subsequent evolution experiments. We are in the process of comparing stability of marked and unmarked versions of plasmid pRGM1.

1.b. Validate plasmid stability measurements using the flow cytometer.

We define plasmid stability as the ability of plasmids to persist within a bacterial population for a given period of time. We aimed to see if the presence of plasmids pB10::*gfp* and pRGM1::*gfp* could be assessed (i) by measuring fluorescence, and (ii) by using flow cytometry instead of the traditional and more time-consuming plate counting methods. As a direct comparison between the two methods, we calculated and compared the fraction of plasmid-containing cells within a bacterial population (i) by counting the numbers of fluorescent (plasmid-containing) and non-fluorescent (plasmid-free) colony forming units (CFU) on LB agar plates containing 100-200 CFUs per plate, using a blue light source (450 nm), and (ii) through flow cytometry using a BD FACSaria III fluorescently activated flow cytometer (hereafter often briefly called 'FACS'), which counts the number of fluorescent (plasmid-containing) and non-fluorescent (plasmid-free) cells directly in the liquid cultures.

Flow cytometry: methods

To measure the presence and the fraction of plasmid-bearing cells using FACS, bacterial cells were harvested from 1 mL cultures by centrifugation and re-suspended in an equal volume of phosphate buffered saline (PBS) to remove potential autofluorescent components. Each cell suspension was diluted 10 to 20-fold before analysis. Plasmid-containing cells were differentiated from plasmid-free cells based on fluorescence intensity (FITC) after excitation with blue light (488 nm). The discriminatory gates were set each day using a positive (plasmid-containing) and negative (plasmid-free) control for each strain, such that the gates accounted for 99.5% of the plasmid-containing cells in the positive control. The photomultiplier voltages to measure the forward scatter (FSC), side scatter (SSC), and fluorescence (FITC), as well as the flow rate were adjusted as necessary. This was also done to yield an event threshold (*i.e.*, number of cells counted) between 800 and 1400 events (=cells) per second. Between 10,000 and 100,000 events were counted for each sample, depending on the bacterial densities.

Flow cytometry: results

During the first 6 months we showed that (i) GFP fluorescence can be used as a proxy for the presence of pB10::*gfp* in *A. baumannii*, since the stability dynamics of the marked and unmarked plasmids were very similar, as determined by fractions of fluorescent and antibiotic resistant colonies (Fig. 1), and (ii) flow cytometry was a reliable high-throughput method to assess the fraction of plasmid-bearing (fluorescent) cells (Fig. 2, 3), as the observed dynamics of plasmid loss in *A. baumannii* were very similar when compared to the traditional plate counting method (Fig. 3).

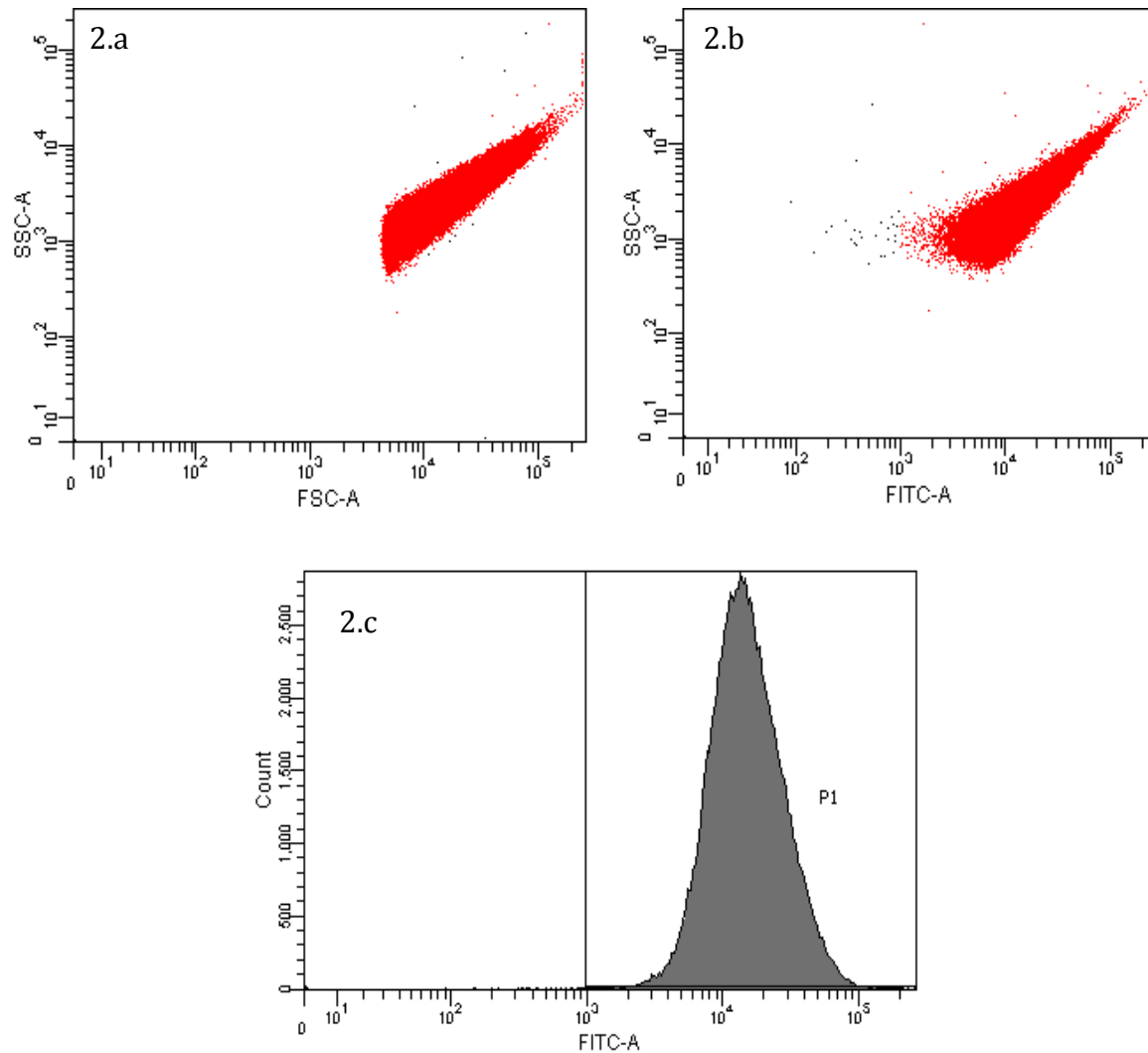


Figure 2. Flow cytometry graph showing the gated fluorescent population of *A. baumannii* ATCC 17978 (pB10::*gfp*) at T₀ (inoculation day). (2.a) side scatter area (SSC-A) versus forward scatter area (FSC-A); (2.b) SSC-A versus fluorescence area (FITC-A); (3.a) cell count versus FITC-A.

Only recently we determined that even for *A. baumannii* pB10::*gfp*, not all antibiotic resistant cells were fluorescent after two weeks cultivation in biofilms or 50 days in serial batch cultures. This could be due to partial integration of the plasmid in the bacterial chromosome, deletion of a plasmid segment that contains the *gfp* gene, mutations in *gfp* or its promoter, or other factors affecting expression and folding

of GFP (see section 1.d 'Biofilm plasmid stability assays' and Fig. 9). We are in the process of testing these hypotheses. We have had more difficulty in using flow cytometry on *Enterobacter aerogenes* ATCC 13048 and *E. coli* ATCC 8739, where fluorescence seemed to decline very rapidly, even when cultures were grown in selective medium (data not shown).

1.c. Test stability of the two plasmids in multiple strains in liquid serial batch cultures in the absence of antibiotics.

Introduction

For each of the two model MDR plasmids (pB10 and pRGM1), we aimed to determine the evolution of plasmid stability in two distinct hosts, which were chosen using the following criteria: (i) their ability to grow in the same mineral salts medium supplemented with the same carbon source, (ii) ability to form biofilms in flow cells, and (iii) ability to replicate plasmids pB10::*gfp* and pRGM1::*gfp*, yet poorly maintain them, and adequately express the green fluorescence encoded on the plasmids. All strains listed in Table 1 were able to grow in liquid MBM mineral salts medium supplemented with 18.5 mM succinate as carbon source. This modified M9 medium, supplemented with trace elements and vitamins, was used previously for biofilm flow cell studies with *E. coli* by our group (Ponciano *et al.*, 2009). The only difference was the use of succinate instead of glucose as a carbon (C) source (at the same C/N ratio), because the *Acinetobacter* strains did not grow with glucose as a C-source.

Plasmid stability assays in liquid cultures: methods

The stability of the ancestral plasmid pB10::*gfp* was determined in several candidate hosts to identify strains in which the plasmid is poorly maintained. Our model plasmids were introduced in the selected strains either by conjugation (plate matings) or

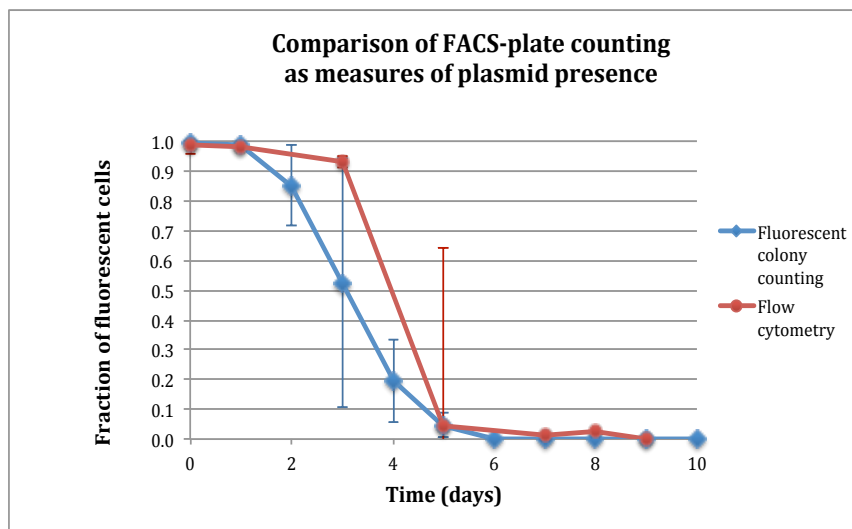


Figure 3: Stability of plasmid pB10::*gfp* in host *A. baumannii* ATCC 17978 in liquid batch cultures, measured by fluorescent colony counting (blue) and flow cytometry (red). Error bars represent standard deviations; some are hidden behind symbols.

electroporation. For plate matings we used *E. coli* AT1036 (pB10::*gfp*) as a donor strain, which is a mutant that requires diaminopimelic acid (DAP). Transconjugants containing pB10::*gfp* were selected on LB agar media without DAP (inhibiting donor growth) and supplemented with Tc (tetracycline) and Km (kanamycin) (preventing growth of plasmid-free recipients). Transconjugants that exhibited fluorescence mediated by the acquisition of the *gfp*-marked plasmid were selected, and presence of the plasmid was verified by plasmid extraction followed by restriction enzyme digestion and agarose gel electrophoresis.

Plasmid stability assay in liquid cultures: results

The stability of pB10::*gfp* was tested in several strains. Plasmid pB10::*gfp* was shown to be rapidly lost in populations of *A. baumannii* ATCC 17978 based on fluorescence (Fig. 3). This result was corroborated by the very low fraction of resistant cells detected by plate counting after 7 days (4×10^{-7} or below detection limit). Additionally, analysis of the plasmid content of selected clones from day 10 through plasmid extractions and gel electrophoresis confirmed the absence of pB10::*gfp*. In conclusion, this *A. baumannii* strain poorly maintains pB10 in a well-mixed liquid system, and therefore is a suitable candidate for plasmid persistence tests in biofilms and for coevolution experiments.

Plasmid pB10::*gfp* was also found to be unstable in *Escherichia coli* ATCC 8739 but plasmid loss did not proceed as rapidly (data not shown). Later results indicated that loss of fluorescence was not consistent with plasmid loss in this strain. We are currently determining whether fluorescence can be used as a suitable proxy for plasmid presence, and whether this strain will be a good candidate as second host. Therefore, we do not show the stability results here. A similar problem of loss of fluorescence without consistent loss of plasmid was observed for *Enterobacter aerogenes* ATCC 13048 (data not shown). Additionally, we have successfully transferred pBP10::*gfp* via mating into *Pseudomonas aeruginosa* PA14. However, due to its natural fluorescence, this strain was not suitable for fluorescence-based analyses. In spite of the fluorescence problems we will consider using one of these strains as plasmid hosts in Year 2 by employing methods that do not rely on fluorescence, such as quantitative real-time PCR.

Also problematic was *Klebsiella pneumoniae* subsp. *pneumoniae* ATCC 700721, which proved to be naturally resistant to both Km and Tc, the two antibiotics used to select for the presence of pB10::*gfp*. Since we had already identified one suitable *A. baumannii* candidate, strain ATCC 17978 described above, we did not further pursue studies with *A. baumannii* BAA-1709. We will continue to screen for a second appropriate host to be used in this project

Studies with plasmid pRGM1 have been delayed due to problems described below (see 'Problem areas' section). We have successfully marked the plasmid but still need to test its stability in some of the hosts listed in Table 1.

Table 1: Strains used in the project

Organism (genus/species)	Strain	Status of genome sequence	Genome size (Mbp)	Number of plasmids (names)	Plasmid sizes	Origin	Source	Resistance to ¹		Biofilm formation	pB10::gfp ²	
								Tc	Km		Presence	Stability
<i>Acinetobacter baumannii</i>	ATCC 17978	Complete	4	2 (pAB1, pAB2)	13k, 11k	Fatal meningitis	ATCC	no	no	good	+	U
	BAA-1709	Complete	3.48	3 (p1ABSDF, p2ABSDF, p3ABSDF)	6.1k, 25k, 24.9k	Gut of louse	ATCC	no	no	moderate	+	TBD, NR
<i>Escherichia coli</i>	ATCC 8739	Complete	4.75	0	NR	Feces	ATCC	no	no	poor	+	M ³
<i>Enterobacter aerogenes</i>	KCTC 2190 (=ATCC 13048)	Complete	5.28	0	NR	Sputum	ATCC	no	no	moderate	+	U ³
<i>Klebsiella pneumoniae</i>	MGH 78578 (=ATCC 700721)	Complete	5.69	5 (pKPN3-7)	175k, 107k, 88.5k, 4.2k, 3.4k	Sputum	ATCC	yes	yes	robust	TBD ⁴	TBD
<i>Pseudomonas aeruginosa</i>	PA14	Complete	6.54	0	NR	Burn wound	Ausubel Lab, Harvard	no	yes	good	TBD ⁴	TBD

TBD, to be determined; NR, not relevant

¹ Tc, tetracycline (10 mg/l); Km, kanamycin (50 mg/l)

² +, presence of introduced plasmid pB10::afp confirmed; U/M, plasmid unstable or moderately unstable, respectively

³ These strains showed inconsistency between loss of fluorescence and loss of plasmid

⁴ Because of problems with fluorescence and intrinsic antibiotic resistance, these strains are not useful pB10 hosts

1.d. For strains showing low plasmid stability in liquids, test plasmid stability in biofilms.

There are at least two characteristics of biofilms that are expected to promote the persistence of drug resistance plasmids compared to well-mixed bacterial populations. First, the absence of global competition is expected to reduce the rate at which plasmid-free hosts sweep through the biofilm population after initial plasmid loss, thus positively affecting overall plasmid persistence. Second, adjacent immobilized cells are expected to show high conjugative transfer rates due to close contact and a lower frequency of mating pair interruption (Molin and Tolker-Nielsen, 2003; Zhong *et al.*, 2010). More efficient horizontal transfer should in turn increase the frequency at which plasmid-free segregants acquire the plasmid from neighboring plasmid-bearing cells, thus promoting overall plasmid persistence. However, there is limited evidence for improved plasmid transfer, and we did not observe high transfer rate of our model plasmid pB10 in previous biofilm studies (Król *et al.*, 2011; Król *et al.*, 2013). Here our biofilm studies are designed to test the hypothesis that our MDR model plasmids are more persistent in biofilm populations as compared to mixed liquid cultures of the same bacterial strain.

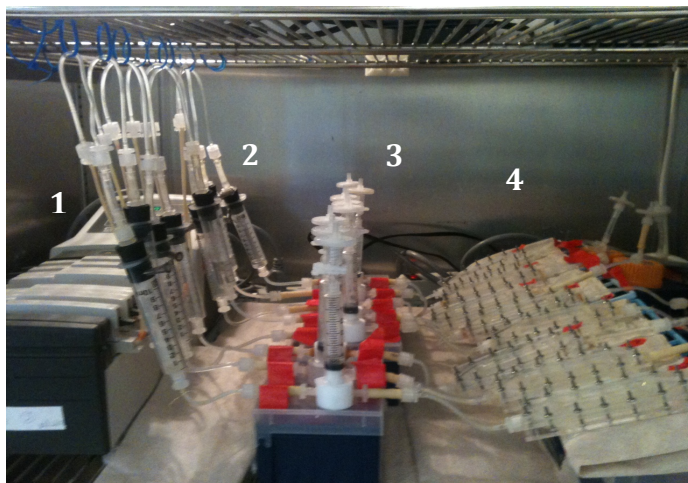


Figure 4. Picture illustrating our biofilm apparatus. A pump (1) pushes growth medium through a flow breaker (2), which impedes the migration of bacteria upstream. A bubble trap (3) collects air that may disturb the biofilm, which is grown in flow cells (4).

Biofilm stability assay: methods

The candidate strains listed in Table 1 were first tested for their biofilm formation capacity in flow cells as described in our previous study (Ponciano *et al.*, 2009). Briefly, the biofilm cultivation system consists of seven parts: (i) medium reservoir, (ii) multichannel pump (iii) bubble trap (iv) flow cell, (v) outflow reservoir, (vi) air pump and (vii) flow breaker (parts iii, iv, v and vii are shown in Fig. 4). The flow cell is made of acrylic plastic. It is constructed from two rectangular plates that are 104 by 48 mm in size. Sidewalls (62 by 26 by 5 mm) were glued to the top plate to form an elongated hexagonal growth chamber. There are 56- by 20-mm square openings in the top and bottom of the rectangular plates that are sealed with 60- by 24-mm glass slides. The upper and lower plates were assembled using screws and sealed using a microseal film. To prepare the inocula, the bacteria were grown overnight, and 200µl aliquots were inoculated into the flow cell, and incubated for 24 hours without the addition of fresh medium. Upon resuming media flow, biofilms were incubated at 25 °C for 22 days. Biofilm formation was evaluated qualitatively based on the observed growth on the glass slide (Table 1). Examples of flow cells with bacterial biofilms are shown in Fig. 5.

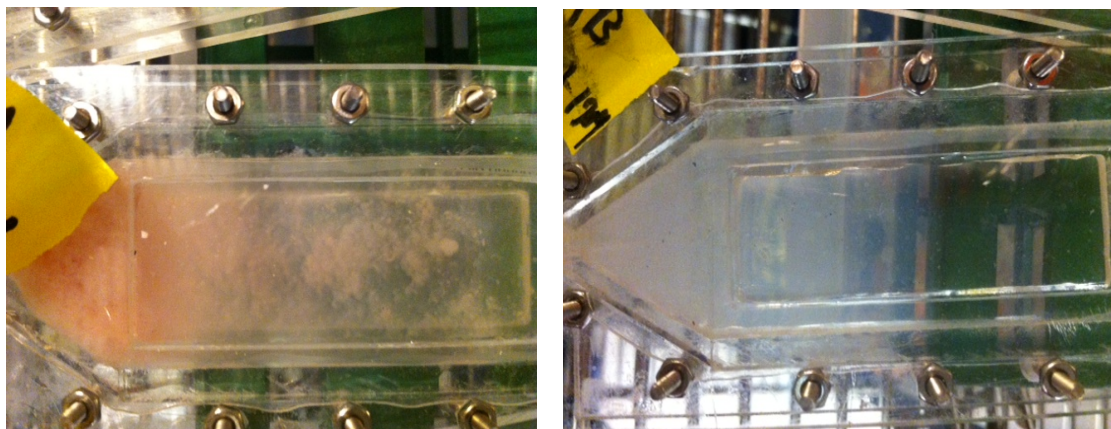


Figure 5: Examples of 22-day old bacterial biofilms, grown at 25°C in flow cells supplied with MBM medium with 18.5mM succinate: *K. pneumoniae* 700921 (left) and *A. baumannii* BAA-1709 (right).

Plasmid stability assays in biofilms were initiated by inoculating each flow cell with cultures of plasmid-bearing clones, which were grown overnight under antibiotic selection to ensure plasmid presence in 100% of the cells. The inoculated flow cells were incubated for 24 hours without addition of fresh medium to allow the bacteria to attach to the surface. The flow of the medium - without supplementation of antibiotics - was subsequently started at a constant rate of 5.2 ml/h and the biofilm flow cells were incubated at 37 °C for 14 days. This change in temperature from 25 °C for the initial biofilm growth tests (Fig. 4) was deliberate because of the growth optima of the strains and consistency with all experiments in liquid batch cultures. At the end of the experiment, the biofilm was immobilized using 3% alginate followed by 60mM CaCl₂. Each biofilm was aseptically sampled using the technique shown in Fig. 6 and described previously (Ponciano *et al.*, 2009). A small fraction (10 µL) of each core was immediately diluted and plated on selective and non-selective media, 500 µL aliquots were centrifuged, re-suspended in an equal volume of PBS, and analyzed using FACS; the remainder of the samples (~500 µL) were archived at -70 °C.

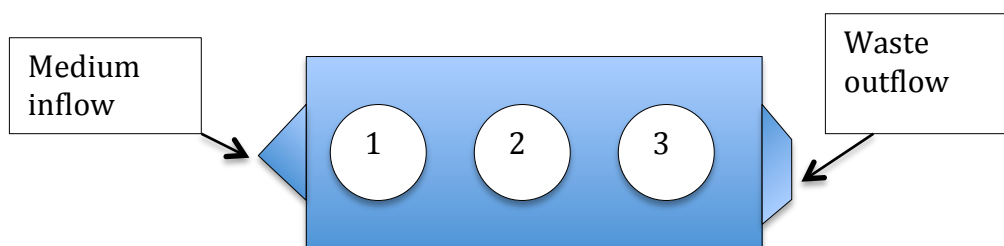


Figure 6: Representation of cores (1-3) taken from a biofilm grown in a flow cell, where fluid dynamics are controlled.

After 24 hours, several clones were tested for phenotypes such as resistance, fluorescence, colony morphology, and size. To determine the identity of strains showing different phenotypes, colony polymerase chain reaction (PCR) using 16S rRNA universal

primers followed by DNA sequencing was performed. This is one way in which we were able to discern if any contaminants were present in the biofilm populations. Moreover, PCR assays were conducted using a variety of plasmid-specific primers to confirm the presence of the plasmid under study. To test the heritability of the fluorescent and resistant phenotypes, a subset of fluorescent and non-fluorescent colonies with different phenotypes were picked and re-plated on selective plates, grown in selective liquid medium, archived, and further analyzed to confirm plasmid presence by plasmid extractions followed by restriction fragment analysis. These steps allowed us to detect complete loss or major structural changes in the plasmid (large insertions, deletions, and chromosomal integration) that might be correlated with the observed phenotypes.

Biofilm plasmid stability assays: results

To date we have conducted replicate biofilm flow cell experiments to test the stability of plasmid pB10::*gfp* in *A. baumannii*, *E. coli* ATCC 8739, and *E. aerogenes* ATCC 13048 when grown in biofilms. Duplicate biofilms of *E. aerogenes* ATCC 13048 were grown in flow cells for 27 days. As in liquid cultures, loss of fluorescence was observed, but it did not correlate with loss of antibiotic resistance. One biofilm replicate retained plasmid-encoded resistance to Tc/Km (Tc^RKm^R), while the other biofilm replicate demonstrated a loss of resistance in approximately half of the clones tested. Because of these problems, we did not yet further pursue *E. aerogenes* as a model host for this study.

After encountering multiple problems with flow cell leakage and contamination (see section 'Problem areas'), we were able to test the stability of pB10::*gfp* in *A. baumannii* biofilms. The fraction of fluorescent (and thus plasmid-bearing) cells was determined for the inoculum (T₀) and on day 14 (T₁₄), using both fluorescent colony counting and flow cytometry (Fig. 7). For cores 1 and 2, the FACS data were surprisingly lower than the plate count data. This issue is still not completely resolved. Compared to the plasmid stability in liquid cultures, where less than 1 in 10⁶ cells were still fluorescent or resistant after 10 days, pB10::*gfp* seems to have persisted slightly longer in host *A. baumannii* in a biofilm after two weeks. Indeed, the percentage of fluorescent cells averaged over the three cores was 4.2% based on plate counting, and 0.4% based on FACS (Fig. 7, 8), yet the numbers of resistant clones was not significantly different from total numbers (data not shown). For all fluorescent *A. baumannii* clones tested, the

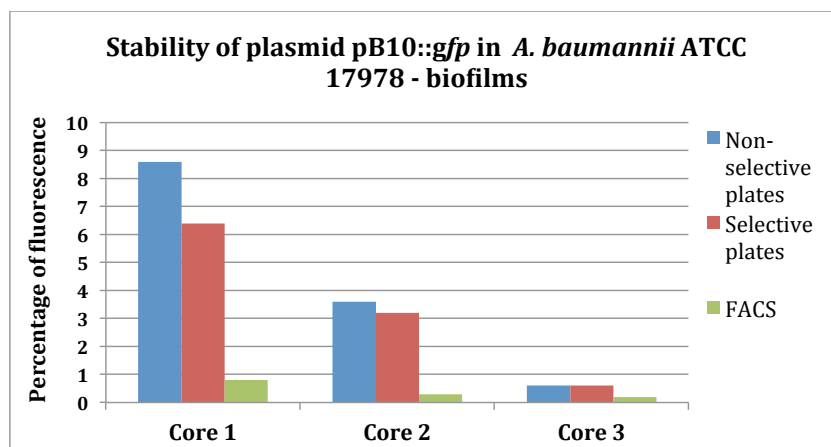


Figure 7. Results of *A. baumannii* ATCC 17978 (pB10::*gfp*) biofilm stability assay for three cores of one biofilm (see Fig. 6). Data represent percentage of fluorescent cells as determined by plate counting on selective (Tc₁₀ and Km₅₀) and non-selective plates, as well as by FACS. Y-axis ranges from 0 to 10% only. The experiment was recently repeated and future reports will present averages and standard errors.

presence of pB10 was assessed, as well as the retention of antibiotic resistance, thus confirming that positive detection of fluorescence is a good measurement for plasmid presence in this host. However, lack of fluorescence was shown not to be a good proxy for lack of plasmid or antibiotic resistance genes, as described below. All clones were confirmed to be *A. baumannii* based on 16S rRNA sequencing.

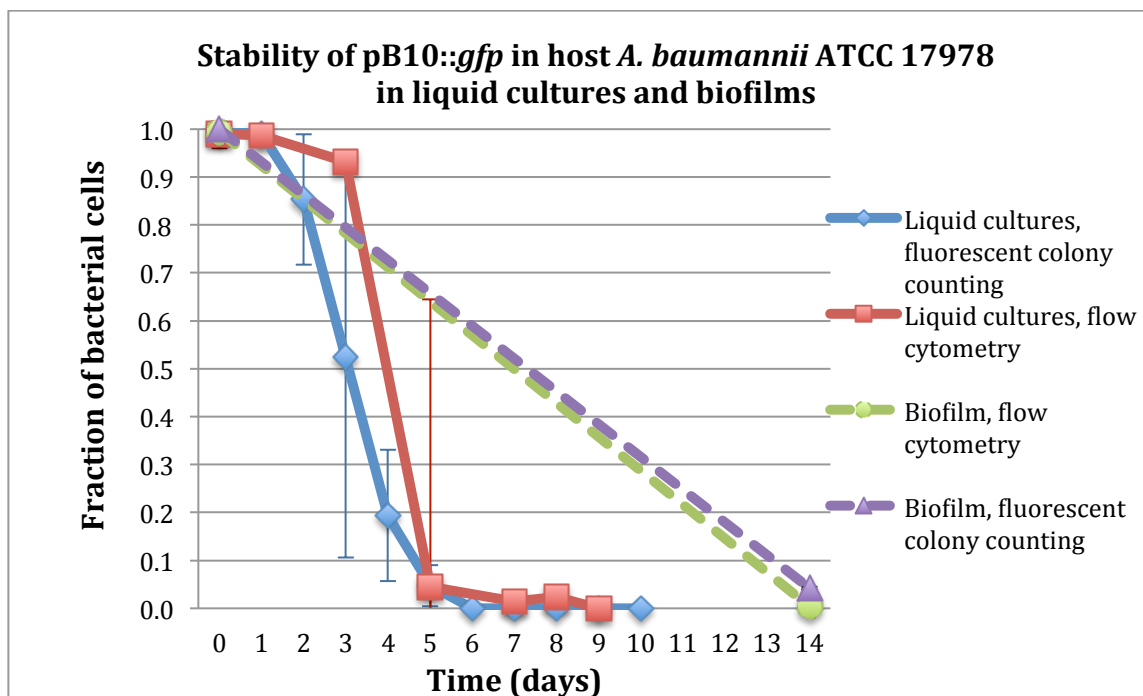


Figure 8: Stability of plasmid pB10::gfp *A. baumannii* ATCC 17978 grown in a biofilm (green) compared to previous results from liquid cultures (red & blue). The data are based on fluorescence, measured by fluorescent colony counting (blue, purple) and flow cytometry (red, green). Error bars represent standard deviations (sometimes hidden under symbol). The red and blue data sets are the same as in Fig. 3. The value of the (green) flow cytometry data point for the biofilm on day 14 was 0.4%.

Analysis of plasmids from fluorescent and non-fluorescent biofilm clones showed different pB10 banding patterns (Fig. 9, 4-12), which were consistent with the observed phenotypes:

- Fluorescent clones (~1-9 % of total, see Fig. 7) were always Tc^RKm^R. They always showed a plasmid of the expected size for pB10::gfp, suggesting that pB10::gfp was intact, as expected (Fig. 9, lanes 9-11).
- Non-fluorescent clones (~91- 99% of total clones, see Fig. 7) were either sensitive or resistant to the two antibiotics. The sensitive clones (2 out of 10 colonies screened; Fig 9, lanes 5 and 8) did not show a plasmid other than their native plasmids, suggesting that plasmid pB10 was no longer present, as expected.
- Interestingly, clones that were non-fluorescent but still Tc^RKm^R had a plasmid that was clearly smaller than pB10::gfp (Fig. 9, lanes 3-4, 6-7; 8 out of 10 non-fluorescent colonies screened). This suggests that the plasmid has sustained large deletions; this

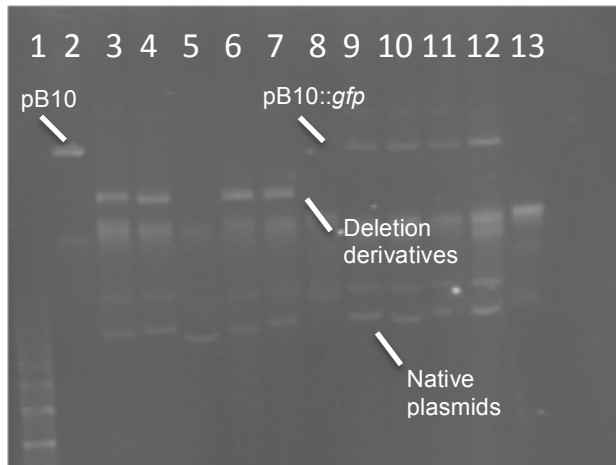


Figure 9: Agarose gel of plasmid DNAs extracted from *A. baumannii* ATCC 17978 (pB10::gfp) biofilm clones and controls. (1) 1 Kb Plus DNA ladder; (2) plasmid pB10; (3-8) non-fluorescent biofilm clones, with 5 and 8 being Tc and Km sensitive and the others being resistant; (9-11) fluorescent Tc^RKm^R biofilm clones; (12) *A. baumannii* (pB10::gfp); and (13) *A. baumannii* ATCC 17978 without pB10::gfp.

was unexpected given previous plasmid analyses of clones sampled at the end of the liquid batch culture stability assays. We still need to confirm which plasmid segments were deleted. We expect that they contained the *gfp* gene, which would explain loss of fluorescence.

Comparison of plasmid stability in liquid cultures and biofilms: results

MDR plasmid pB10::gfp was poorly maintained in liquid cultures of *A. baumannii*, based on both fluorescence and resistance measurements (Fig. 8). Although not visible in Fig. 8 because of the linear scale, after 10 days the fraction of resistant or fluorescent cells was lower than 10^{-6} , indicating very rapid loss of the plasmid in the well-mixed population. However, in biofilms, while fluorescence was also lost albeit less rapidly, resistance was lost even

more slowly. Indeed, in two of the three biofilm cores, the number of resistant clones was not significantly different from the number of total clones (data not shown). Replica-plating of individual colonies from non-selective to selective plates also showed that only 20% of the non-fluorescent clones had lost resistance. This has important implications for the spread and persistence of antibiotic resistance genes. Ultimately, the question is not so much whether the plasmid remains intact, but whether the resistance genes persist in these biofilm populations and can still spread to other pathogens. We are still assessing the molecular mechanisms that allowed the resistance genes to be maintained while fluorescence was lost, but speculate that a plasmid segment containing the *gfp* gene was deleted. We are currently testing if these smaller deletion variants are still transmissible at the same rate as the wild-type plasmid. Our preliminary conclusion is that the plasmid and the plasmid-encoded resistance determinants are more readily maintained in the structured biofilm environment than in the liquid batch cultures (Fig. 8). This supports our hypothesis that the rate at which plasmid-free cells can sweep through the structured population is lower than in well-mixed liquids. These findings raise the concern that antibiotic resistance likely persists longer in bacterial biofilms formed in wounds than in planktonic populations of the same bacteria, thus threatening effective wound treatment.

Moreover, it is becoming apparent that loss of fluorescence during long-term cultivation confounds the fluorescence measurements employed as a proxy for plasmid presence. Recent results of repeated biofilm experiments confirmed our findings. This is interesting from an evolutionary perspective, as purifying selection seemed to act on traits that are not advantageous and likely deleterious (*i.e.*, fluorescence), while the antibiotic resistance genes under study were maintained more easily.

Task2: Characterize the evolution of plasmid stability in bacterial hosts grown in biofilms and well-mixed liquid cultures.

The main goal of this project is to identify the pathways by which MDR plasmids evolve improved stability in a novel host. Addressing this question requires that we understand whether the mechanisms involved are 1) host-dependent, 2) plasmid-dependent, or 3) both. To this end, we co-evolved the host bacterial strain with its chosen model plasmid under antibiotic selection and will analyze the phenotype and genotypic changes that may explain improvement of plasmid persistence.

Task 2 is further divided into two sections: 'Evolution experiments' (sections 2.a-2.d) and 'Comparison of stability patterns' (sections 2.e-2.j), as described below.

2.a. Evolve host A with first plasmid [A (pB10::gfp)] under two conditions (biofilm, liquid).

Having confirmed the instability of plasmid pB10::gfp in host *A. baumannii*, we proceeded to coevolve this host-plasmid pair in serial liquid batch cultures under antibiotic selection.

Coevolution experiment: methods

Prior to commencing the evolution experiments, we selected for a rifampicin resistant (Rif^R) and a nalidixic acid resistant (Nal^R) *A. baumannii* mutant. This allows us to select and counterselect evolved and ancestral hosts at a later time. We introduced pB10::gfp into each of these strains via conjugative mating, and briefly pre-adapted them to MBM-succinate medium with respective antibiotics. The coevolution experiment in liquid serial batch cultures with *A. baumannii* (pB10::gfp) was carried out as described by De Gelder *et al.* (2008). Briefly, five lineages of a Rif^R *A. baumannii* (pB10::gfp) were coevolved for 50 days (corresponding to about 500 bacterial generations), by growing and passaging 4.9 µL of each culture in selective liquid medium, while incubating overnight at 37°C in a shaker. The host without the plasmid was also evolved in parallel as a control to identify mutations that may occur independently of coevolutionary pressures. The number of fluorescent, antibiotic resistant, and sensitive CFU was recorded at the beginning, end, and throughout the coevolution experiment using FACS and plate counting. Moreover, a subset of clones representing the different phenotypes (*i.e.*, color, size, shape, antibiotic resistance, *etc.*) was further analyzed to determine the following: (i) to confirm identity of the host strain via 16S rRNA gene sequencing (to detect any eventual contamination), (ii) to confirm plasmid presence by PCR using plasmid-specific primers and plasmid extractions, (iii) to determine restriction fragment length patterns of extracted plasmid DNA to detect any major structural rearrangements, and (iv) transfer the evolved plasmids to another host (such as *E. coli* K12Nal) to see if they are still able to replicate in that host and confer antibiotic resistance.

Post-coevolution stability assay: methods

To determine if coevolution resulted in improved plasmid stability, we have started to compare the stability of pB10::*gfp* in 20 randomly chosen evolved clones isolated from each of the 5 coevolved *A. baumannii* (pB10::*gfp*) lineages with that of 3 of their ancestral (*i.e.*, not coevolved) strains. For this set of stability assays, we passaged cultures in deep-well plates for 10 days. Cultures were grown in 1.5 mL of MBM-succinate medium, and incubated in a plate shaker at 37 °C overnight. Because loss of fluorescence was a concern, samples were plated every other day to count the fraction of fluorescent (when possible) clones throughout the assay. We are currently in the process of evaluating plasmid stability in a total of 100 clones (20 from each of the 5 coevolved lineages) and comparing it to that of their ancestor. The following scenarios are possible, and will determine our subsequent research methodology:

- I. If the evolved plasmid is equally stable in both ancestral and evolved hosts, then any adaptive change linked to increased stability is likely to be located on the evolved plasmid.
- II. If the stability of the evolved plasmid is not the same, then we will conduct additional stability assays where the stability of the ancestral plasmid in the evolved host (cured of its co-evolved plasmid) will be compared to that of both the ancestral host-plasmid pair and that of the evolved host-plasmid pair.

This will result in one of three possible outcomes:

1. If the stability of the ancestral plasmid in the evolved host is equal to that of the evolved host-plasmid pair, then advantageous mutations associated with improved stability must be on the genome of the evolved host.
2. If the stability of the ancestral plasmid in the evolved host is as poor as in the ancestral host, then selection is most likely not acting on the host alone.
3. If the stability of the evolved plasmid in the ancestral host is somewhat greater than that of the ancestral plasmid in ancestral host, but not as stable as in the co-evolved host-plasmid pair, then advantageous mutations associated with plasmid stability occurred in both the coevolved host and plasmid; thus, stability-related traits are under selection in both.

Sequencing of multiple host genomes and plasmid DNA will follow as per our SOW.

Evolution experiments: results

We successfully completed a 50-day coevolution study in liquid cultures for *A. baumannii* Rif^R (pB10::*gfp*). Throughout the assay, at intervals of about 100 generations, we verified presence of pB10 by PCR using primers that amplify three regions on the plasmid (including segments of genes *trbC*, *trfA*, and *kfrA/korB*). Moreover, we confirmed the identity of selected clones using 16S rRNA sequencing. After 100 generations, we also successfully transferred pB10 from lineage #4 (which had lost fluorescence) into *E. coli* K12, which was able to transfer Tc and Km resistance, indicating that the plasmid still had those genes and was still self-transmissible, despite the loss of fluorescence. As shown in Fig. 10, the fraction of fluorescent CFUs for two of the five lineages (Lineages # 1 and 2)

remained approximately 1 for the duration of the experiment. However, in the three other lineages that fraction rapidly decreased at different time points (Fig. 10). Given the loss of fluorescence but the maintenance of resistance, as shown by the ability of each colony to grow in the presence of antibiotics, we will continue to assess physical plasmid presence and antibiotic resistance in addition to fluorescence. We are in the process of characterizing potential structural changes in the evolved plasmids, just like we did for the biofilm clones (Fig. 9). Preliminary results suggest that again, at least two plasmids variants were found: intact plasmids and deletion variants. Complete genome resequencing of clones that show unique plasmid patterns will provide detail information in the coming months.

Post-evolution stability assay: results

To determine if plasmid stability improved after plasmid-host coevolution, we have begun to assess the stability of 20 coevolved plasmids in their co-evolved hosts from Lineage #2. As illustrated in Fig. 11, using FACS, we determined that the fraction of fluorescent cells among clones varied between 0.15 to 0.9 at T_0 , between 0.25 and 1 at T_5 , and between 0.3 to 1 at T_{10} . The low fraction at T_0 in some clones was unexpected as clones were

isolated from a still fluorescing Lineage (#2) and were grown in the presence of antibiotics; they should thus have retained their fluorescence. In spite of this variability that needs to be addressed through adjustment of FACS settings, the data show that there is no clear loss of fluorescence over time during the stability assay in the absence of antibiotics (T_{10} versus T_0). Moreover, the fraction of resistant cells was still above 0.35 (with the exception of clone 20), suggesting that the plasmid - or at least a segment that contains the *gfp* and resistance genes - was successfully retained in the absence of selection. These results were in stark contrast with the poor retention of the ancestral plasmid in the ancestral plasmid-host pair (A1-3) (from ~ 1 to < 0.01 by day 10), as expected from previous stability assays (Figs. 1,3,8). This finding suggests that when *A. baumannii* is allowed to co-evolve with a recently acquired MDR plasmid under antibiotic selection, persistence of the plasmid-encoded resistance genes can dramatically improve within 500 generations (here 50 days).

The evolution experiment in replicate biofilm flow cells is currently underway and will be analyzed in the coming weeks.

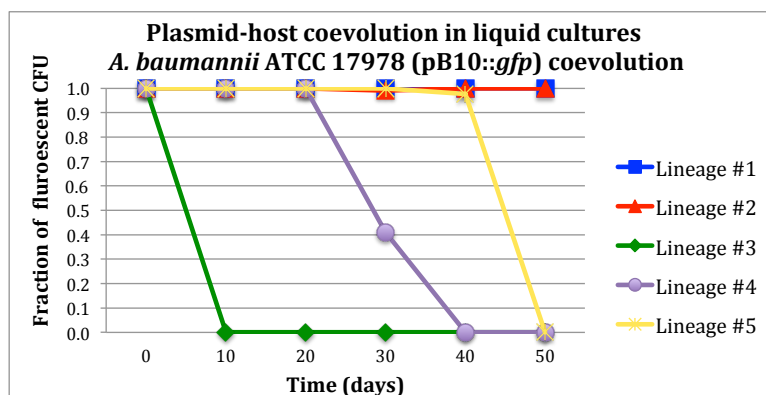


Figure 10. Temporal dynamics of the fraction of fluorescent cells (based on fluorescent and total colony forming units (CFU) on LBA) during 500 generations (50 days) of plasmid-host coevolution for each of the five lineages of *A. baumannii* ATCC 17978 (pB10::gfp).

Other tasks under Task 2 as per SOW

Tasks **2.b-2.g** have not yet been addressed. However, we will soon determine the stability of the second plasmid (pRGM1) in host *A. baumannii*.

Tasks **2.h-2.j** were scheduled for year 2, as was most of Tasks 3 and Task 4.

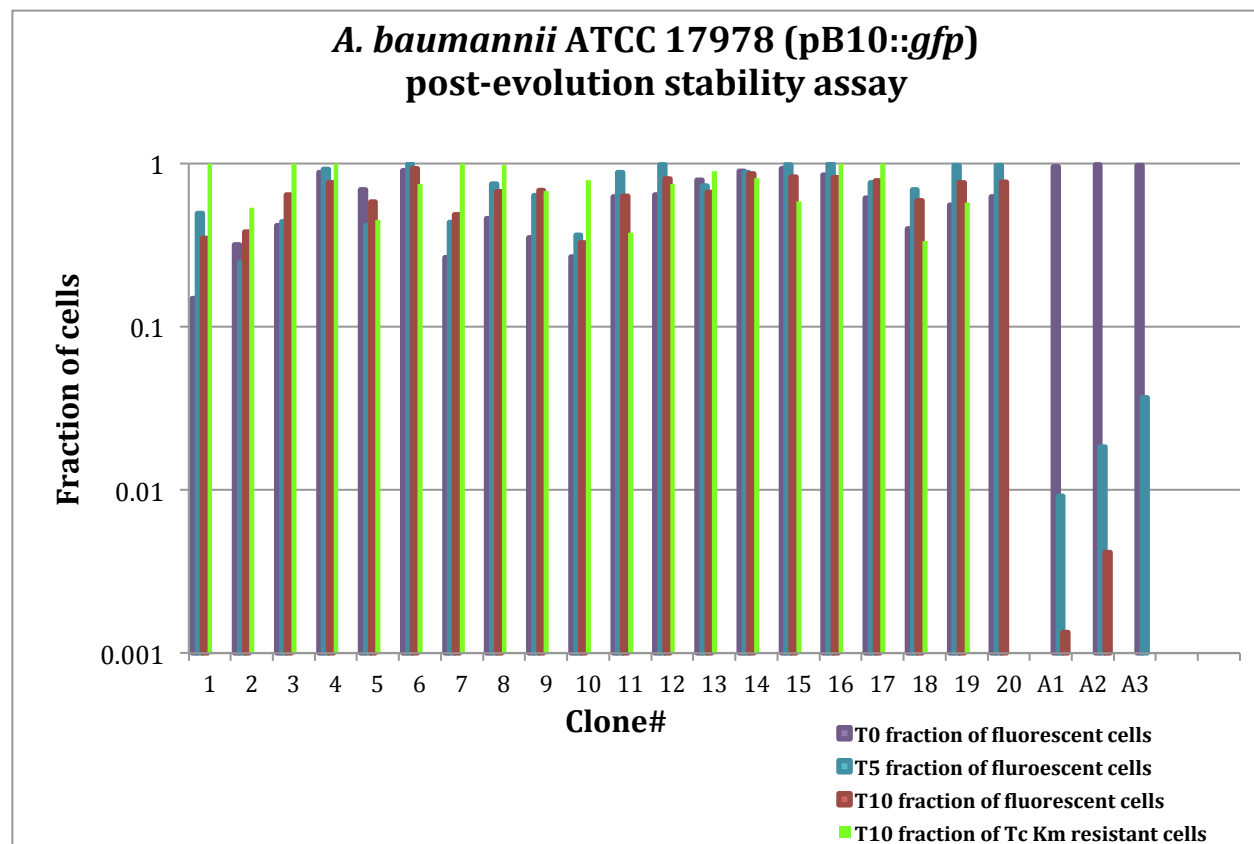


Figure 11. Stability of pB10::*gfp* in its co-evolved *A. baumannii* ATCC 17978 Rif^R (clones 1-20) from Lineage #2 compared to stability of ancestral plasmid in ancestral host (A1-3). Data represent the fraction of fluorescent cells assessed with FACS at days 0, 5 and 10 (T₀, T₅, T₁₀), as well as the fraction of antibiotic resistant cells at T₁₀ (green). The results clearly show improved retention of fluorescence and resistance after evolution compared to the ancestral plasmid-host pair.

Problem areas

While we have met several of our targets during the course of the first year of this two-year project, our progress has been hindered by technical challenges, many of which have been resolved. What follows is an explanation of the difficulties we have encountered, listed under one of three following categories: 1) GFP and flow cytometry, 2) assessing growth rates in biofilms, and 3) biofilm assays.

1) GFP and flow-cytometry related difficulties

Making plasmid constructs

Employing a green fluorescent protein (GFP) as a proxy to detect plasmid presence is in principle a remarkable idea, which has the potential of allowing a high-throughput screening of up to 100,000 bacterial cells in a few minutes. While plasmid pB10 was successfully marked with the *gfp* gene previously (van Meervenne *et al.*, 2012), marking plasmid pRGM1 has been very difficult. During the first and second quarter, we attempted to construct a marked version of plasmid pRGM1 as we did for pB10 using transposon mutagenesis (Van Meervenne *et al.*, 2013). We interrogated randomly selected clones for mini-Tn5-P_{A1-04/03}::*gfp* insertion, and found that they were false positives displaying the correct phenotype. We subsequently used new donor strains for the transposon delivery vector, such that the marked plasmids are self-transmissible by conjugation. Unfortunately, this process was also not without setbacks. Finally, multiple variants with unique insertion positions of the mini-transposon were obtained, based on analysis of restriction fragment length patterns of plasmid DNA. We are currently verifying the exact insertion locations by determining the sequence of the junctions of the insertions.

Flow cytometry

Linked to the expression of GFP is the use of fluorescently-activated flow cytometry (FACS) to assess plasmid presence. After the necessary optimization steps, which took several months of rigorous work, we are now able to benefit from this method's potential to rapidly determine fractions of fluorescent bacteria in a population, and thus generate data on putative plasmid presence/absence in a high-throughput manner. Given the high laser settings necessary to detect small bacterial cells such our *A. baumannii* strain, our analyses were being confounded by the detection of similarly small particles present in our dilution buffer (PBS). These inorganic particles may also autofluoresce, and therefore have the potential of affecting both the fraction of fluorescent and non-fluorescent cells in each sample. This is typically not a challenge when interrogating many other prokaryotic cells that are larger and require lower laser setting. We were able to address this problem by 1) optimizing the laser settings for each bacterial strain, and 2) using three controls for each experiments (a plasmid-free strain, a plasmid-bearing strain grown with antibiotics, and a PBS blank). Another challenge was the wide range of forward scatter (FSC, correlated to size and other cell aspects) and fluorescence (FIT-C) values for each population analyzed. Our analyses have consistently shown a range of about 2 orders of magnitude for FSC and, and 2-5 for FITC. Thus, we conducted several experiments to understand what variables (*i.e.*, cell growth stage, orientation of the rod-shaped *A. baumannii* cells, and cell clumping) determined the wide range of FSC and FITC. We found that variance in these parameters in

our clonal populations at least in part reflects differences in cell physiology due to differences in growth stage, as well as cell clumping. We have tried different techniques to assess and reduce the effects of the latter, and observed a reduction in cell clumping using sonication and addition of SDS prior to FACS analysis.

Irregular loss of fluorescence

One of the inherent difficulties of this project is given by the variety of taxa with which we work. Each strain in fact behaves differently when analyzed by FACS, and moreover can change over time, especially when being coevolved for 50 days with its fluorescently-marked plasmid. As described in Section 1.c above, in some cases [*i.e.* *E. aerogenes* ATCC 13048 (pB10::*gfp*) and possibly *E. coli* ATCC 8739 (pB10::*gfp*)], loss of fluorescence does not correspond to loss of antibiotic resistance and, therefore, possibly of the plasmid. The same problem also applies to *A. baumannii* (pB10::*gfp*) as described in Sections 1.c, and 1.d, where often only 10% of antibiotic resistant clones at the end of a stability assay were fluorescent. We are currently still characterizing this phenomenon, and first indications suggest deletion of a plasmid segment that contained *gfp* gene, as well as possible integration of *gfp* and resistance gene into the bacterial chromosome.

2) Challenges imposed by the inability to directly assess cell growth rates in biofilms

As described in the proposal and in previous reports, one complication we face in this research is the variability of cellular growth rates in bacterial biofilms, and the inability to calculate a meaningful population-wide doubling time. Generally, cells near the biofilm-medium interface grow actively, while those residing within the biofilm matrix experience nutrient depletion and possibly arrested growth. This uneven pattern of cellular growth is in marked opposition to the growth rate of cellular populations grown by serial subculture in well-mixed liquid media. More specifically, in serially passaged liquid cultures, a population experiences extended periods of rapid growth, where the growth rate can easily be measured, and the number of generations that occur in some time interval can readily be determined. This is a nontrivial difference for at least two reasons: first, the principle mechanism of plasmid loss in bacteria is the failure to partition plasmids between daughter cells during cell division. If cells in some regions of biofilms are not growing (dividing), then plasmid persistence might occur even when a plasmid-host pair is inherently unstable. It is thus important to distinguish between the *persistence* and *stability* of plasmids in a bacterial population, where plasmid persistence *per se* cannot be used as a surrogate for plasmid stability. For this reason we have proposed to explicitly test plasmid stability in well-mixed liquid cultures before and after plasmid evolution in biofilms and liquid cultures (Task 2), and thus avoid the complexity of doing so in biofilms. Second, we cannot compare the number of cell doublings in well-mixed liquid cultures to that occurring during the same time interval in biofilms. Thus, we have proposed to serially transfer well-mixed liquid cultures and grow bacterial biofilms for a similar number of days. While not ideal, it is the best approximation we can make without compromising the growth of biofilms, while still able to characterize the variance in plasmid stability phenotypes in the two environments. Implementing these solutions, and especially the first one (testing for plasmid stability before and after coevolution experiments) requires

additional weeks of time and resource investment, but greatly improves our methods of inquiry.

3) Difficulties with biofilm assays

During the course of the first several months of this project, we encountered two biofilm-related problems: 1) small leaks in our flow cell set-up, and 2) contamination of biofilm cultures. As explained here, our rigorous experimental set up allowed us to identify and trace the contamination to its source, which we successfully addressed.

More specifically, the biofilm apparatus has numerous components, each of which can potentially present a source of leakage or contamination. As illustrated in Fig. 4, a peristaltic pump pushes the medium into the flow cells; a flow breaker is used to impede upstream migration of the bacteria, and a bubble trap is used to remove gases that may disturb biofilm growth. Waste product is eliminated into a separate container, and a release valve is used to eliminate gases produced by metabolic processes taking place within the flow cell. During the first biofilm experiments lasting 14 days each, leaks were observed at either the bubble trap joint, or from the flow cell themselves. This may have allowed the introduction of other bacterial species through the compromised parts. Alternatively, the contaminants were still present in the tubing, connections, and flow cells from previous use due to incomplete disinfection before the start of the experiments. The routine procedure of running diluted bleach through the entire system was apparently insufficient to eliminate them. While the tubing and most other small components of this system are autoclavable, the flow cells are not.

It should be noted that while several commercial kit options exist for biofilm growth and research, we have not yet found one that is suitable for our experiments, which last up to 50 days, as opposed to the more typical 48-72 hours. Our current set up has been successful to grow *E. coli* biofilms for years in the laboratory of collaborator Dr. Forney, where the biofilms are grown now, but they required further optimization for this project. We successfully addressed the contamination problem by:

- Autoclaving all components, except the flow cells, before assembling them. This includes the waste bottle and waste lines. We replaced the stopcocks with clamps on the outside of the tubes.
- Each replicate biofilm flow cell (typically four) is attached to a self-contained waste bottle that does not have any other flow cells attached to it.
- The concentration of bleach used to sterilize the flow cells is now 10%, and two liters of bleach are being used in a slow overnight disinfection, followed by an additional 2 liters the following day.
- We plate and grow the outflow regularly and conduct 16S ribosomal RNA PCR and sequences on a sub-sample of the bacterial colonies to test for possible contamination without compromising the biofilm.

Since we first experienced contamination, we have implemented the changes described above and have now successfully completed a full plasmid stability assay on *A. baumannii* (pB10::*gfp*). A 50-day coevolution experiment in biofilms is almost halfway.

KEY RESEARCH ACCOMPLISHMENTS:

- We have constructed a marked variant of plasmid pRGM1 (plasmid pB10 was marked previously by our group).
- We have standardized flow cytometry as a high-throughput method to assess plasmid presence using fluorescence as a proxy.
- We have performed plasmid stability assays in well-mixed liquid cultures for several strains (*A. baumannii* ATCC 17978, *E. aerogenes* ATCC 13048, *E. coli* ATCC 8739).
- We have identified two hosts in which plasmid pB10::*gfp* replicates but is poorly maintained, making them good candidates for evolution experiments: *A. baumannii* ATCC 17978 and possibly *E. coli* ATCC 8739.
- We have successfully conducted a first biofilm experiment with *A. baumannii* (pB10::*gfp*) to compare plasmid stability with that in liquid cultures.
 - The plasmid or a variant that still encodes resistance was shown to be retained much better in biofilms than in liquid cultures. **This is an important finding, suggesting that antibiotic resistance is more easily retained in the structured biofilm environment, as originally postulated.**
 - Characterization of plasmids extracted at the end of plasmid stability assays in liquid cultures showed either the presence or absence of pB10, while biofilm clones showed three permutations: plasmid presence, complete plasmid absence (rare), or the presence of a smaller plasmid, consistent with loss of fluorescence but retention of resistance genes. **This suggests that the MDR plasmid pB10 has undergone structural changes during biofilm growth of *A. baumannii* in the absence of antibiotics, thereby retaining at least two of its resistance genes.**
- We have successfully completed a coevolution experiment in liquid serial batch cultures, where five replicate *A. baumannii* (pB10::*gfp*) populations were evolved under antibiotic selection for 50 days.
 - Preliminary results indicate that the plasmid-encoded antibiotic resistance genes are retained at a much higher frequency after evolution. **This suggests that when *A. baumannii* is allowed to co-evolve with a recently acquired MDR plasmid under antibiotic selection, persistence of the plasmid-encoded resistance genes can dramatically improve within just 500 cell doublings.**

REPORTABLE OUTCOMES:

Two posters and one oral presentation summarizing the research funded by this grant undertaken during this first year have been presented at local and regional scientific conferences:

- Regmi R, Smith SE, Millstein JH, Cornea A, France MT, Corona Hernandez D, Forney LJ, Top EM. 2013. Effects of spatial structure on the co-evolution of multi-drug resistance plasmids and their host. Poster presented at the 2013 IBEST Science Expo, University of Idaho, Moscow, ID.
- Smith SE, Loftie-Eaton W, Norton AS, Millstein JH, Top EM. 2013. Assessing plasmid stability in biofilms and well-mixed populations of *Acinetobacter baumannii* using flow cytometry. Poster presented at the Inland Northwest Genomic Research Symposium. Moscow, ID.
- Loftie-Eaton W, Norton AS, Smith SE, Top EM. 2013. Exchanging mundane techniques for measuring plasmid stability in bacteria with high throughput technologies." Oral presentation, Inland Northwest Genomic Research Symposium. Moscow, ID.

Two manuscripts are in preparation and others are being conceptualized as the analyses proceed and results are obtained:

- Loftie-Eaton, W., A. Norton E.M. Top. Fluorescent flow cytometry and Real-Time qPCR methods for measuring plasmid stability in comparison to the traditional method. In preparation.
- Smith S.E., Millstein J.H., Cornea A, Dudis A., Regmi R., France M.T., Forney L.J., Top E.M.. Effects of biofilm growth on the retention of plasmid-encoded antibiotic resistance genes in *A. baumannii* ATCC 17978. In preparation.

CONCLUSION:

Nosocomial infections of combat wounds in military treatment facilities in the US have become a major problem, and are commonly caused by MDR Gram-negative bacteria such as *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*, *Enterobacter sp.* and *E. coli* (Eardly *et al.*, 2011). For example, *A. baumannii* is an important pathogen responsible for outbreaks of nosocomial infections in intensive care units, including those in military hospitals (Adams-Haduch *et al.*, 2008; Dijkshoorn *et al.*, 2007; Gaynes *et al.*, 2005; Peleg *et al.*, 2008). Strains of these species are often resistant to multiple antibiotics as a result of genes encoded on so-called multi-drug resistance (MDR) plasmids they carry. This wide-spread resistance to antimicrobial agents has led to decreasing effectiveness of current antibiotic treatments, so much that the CDC recently declared antimicrobial resistance as "one of our most serious health threats". Moreover, the bacteria in these wounds often grow in biofilms, which are considered 'hot-spots' for plasmid transfer. However, the adaptive mechanisms by which MDR plasmids are able to persist in bacterial populations even in the absence of antibiotics remains poorly understood. Our main goal of this project is to understand how MDR broad-host-range plasmids can become more stable (*i.e.*, persist) in important wound pathogens, thus perpetuating their encoded resistance traits. We postulated that the spatial

structure of biofilms affects the persistence of MDR plasmids, as well as the evolutionary pathways that further improve that persistence. Therefore, we are conducting plasmid stability and evolution studies to assess and compare the effect of spatial structure (or lack thereof) in biofilms and well-mixed liquid cultures.

Our preliminary results indicate that **the structured biofilm environment, which typically characterizes the type of bacterial growth in wounds, facilitates the persistence of MDR plasmids.** Moreover, we have shown that **plasmids coevolved with their host under antibiotic selection for 50 days have improved their persistence in part by undergoing large structural changes.** While still partially uncharacterized, these genetic modifications likely decrease plasmid cost to the host while retaining favorable plasmid-encoded traits (*i.e.* antibiotic resistance). Once these structural changes and other possible mutations are characterized through whole genome resequencing in Year 2, this project will greatly contribute to our understanding of the pathways through which stable plasmid persistence is achieved in wound-like biofilm environments.

The fundamental insights obtained so far will support future research into novel drug therapies that are based on restricting the dissemination and stable replication of MDR plasmids. Such therapies have recently been proposed by others in the field and are rapidly gaining interest given the alarmingly growing ineffectiveness of currently used antibiotics (Baquero *et al.*, 2011; Lujan *et al.* 2007; Williams *et al.*, 2011). Rather than trying to kill the bacteria, these new compounds aim at preventing the emergence of bacteria resistant to the next generation of antibiotics, by inhibiting spread and stable persistence of the mobile elements that carry the corresponding resistance genes. They will ultimately be useful in the care of patients with trauma-induced wounds, and may thus have a significant impact on the mortality rates of wounded troops as well as their ability to return to combat after injury (Eardly *et al.*, 2011).

REFERENCES:

- Adams-Haduch, J. M., D. L. Paterson, H. E. Sidjabat, A. W. Pasculle, B. A. Potoski, C. A. Muto, L. H. Harrison, and Y. Doi. 2008. Genetic basis of multidrug resistance in *Acinetobacter baumannii* clinical Isolates at a tertiary medical center in Pennsylvania. *Antimicrob. Agents Chemother.* 52:3837-3843.
- Baquero, F., T. M. Coque, and F. de la Cruz. 2011. Ecology and evolution as targets: the need for novel eco-evo drugs and strategies to fight antibiotic resistance. *Antimicrob. Agents Chemother.* 55:3649-3660.
- Center for Disease Control and Prevention (CDC), US Department of Health and Human Services. Antibiotic resistance threats in the United States, 2013.
- De Gelder, L., J.M. Ponciano, P. Joyce, and E.M. Top. 2007. Stability of a promiscuous plasmid in different hosts: No guarantee for a long-term relationship. *Microbiology* 153: 452-463.
- De Gelder, L., J. J. Williams, J. M. Ponciano, M. Sota, and E. M. Top. 2008. Adaptive plasmid evolution results in host-range expansion of a broad-host-range plasmid. *Genetics* 178:2179-2190.

- Dijkshoorn, L., A. Nemec, and H. Seifert. 2007. An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. *Nat. Rev. Microbiol.* 5:939-951.
- Eardley, W. G. P., K. V. Brown, T. J. Bonner, A. D. Green, and J. C. Clasper. 2011. Infection in conflict wounded. *Phil. Trans. R. Soc. Lon. B.* 366:204-218.
- Gaynes, R., and J. R. Edwards. 2005. Overview of nosocomial infections caused by gram-negative bacilli. *Clin. Infect. Dis.* 41:848-854.
- Król, J.E., H. D. Nguyen, L. M. Rogers, H. Beyenal, S. M. Krone, E. M. Top. 2011. Increased plasmid transfer in *E. coli* K12 biofilms at the liquid-air interface. *Appl. Environ. Microbiol.* 77: 5079-5088.
- Król, J. E., A. J. Wojtowicz, L. M. Rogers, H. Heuer, K. Smalla, S. M. Krone, and E. M. Top. 2013. Invasion of *E. coli* biofilms by multidrug resistance plasmids. *Plasmid* 70: 110–119.
- Lujan, S. A., L. M. Guogas, H. Ragonese, S. W. Matson, and M. R. Redinbo. 2007. Disrupting antibiotic resistance propagation by inhibiting the conjugative DNA relaxase. *Proc. Natl. Acad. Sci. U. S. A.* 104:12282-12287.
- Molin, S., Tolker-Nielsen, T., 2003. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Curr. Opin. Biotechnol.* 14, 255-261.
- Murray, C. K. 2008. Infectious disease complications of combat-related injuries. *Crit. Care Med.* 36:S358-S364.
- Peleg, A. Y., H. Seifert, and D. L. Paterson. 2008. *Acinetobacter baumannii*: Emergence of a successful pathogen. *Clin. Microbiol. Rev.* 21:538-582.
- Ponciano, J.M., H.J. La, P. Joyce, and L.J. Forney. 2009. Evolution of diversity in spatially structured *Escherichia coli* populations. *Appl. Environ. Microbiol.* 75:6047-6054.
- Van Meervenne, E., E. Van Coillie, F. Devlieghere, L. Herman, L.S.P. De Gelder, E.M. Top, and N. Boon. 2012. Strain specific transfer of antibiotic resistance from an environmental plasmid to foodborne pathogens. *J. Biomed. Biotechnol.* 2012: ID 834598.
- Williams, J. J., E. M. Halvorsen, E. M. Dwyer, R. M. DiFazio, and P. J. Hergenrother. 2011. Toxin-antitoxin (TA) systems are prevalent and transcribed in clinical isolates of *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*. *FEMS Microbiol. Lett.* 322:41-50.
- Zhong, X., J. Król, E.M. Top and S.M. Krone. 2010. Accounting for mating pair formation in plasmid population dynamics. *J. Theor. Biol.* 262: 711–719. (PMCID: PMC2821984).